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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. ET971611288US

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Irene	Karantzeni	Waltham, Massachusettes

X Additional Inventors are being named on the _____ separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

ISOTHERMAL TITRATION CALORIMETRY ASSAYS

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ENCLOSED APPLICATION PARTS (check all that apply)

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[ ] Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

[ ] Applicant claims small entity status. See 37 CFR 1.27.

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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[Page 1 of 2]

Respectfully submitted,

SIGNATURE _____ Date 06/30/03

TYPED or PRINTED NAME Robin S. Quartin

REGISTRATION NO. (if appropriate) 45,028

Docket Number: 100997-1/US

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USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

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Complete If Known	
Application Number	Not yet assigned
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Examiner Name	Not yet assigned
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METHOD OF PAYMENT *(check all that apply)*

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FEE CALCULATION

1. BASIC FILING FEE

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1001	750	2001	375	Utility filing fee	
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1004	750	2004	375	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	160.00

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2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

	Total Claims	Independent Claims Multiple Dependent	Extra Claims below	Fee from below	Fee Paid
		-20** =		X	=
		-3** =		X	=

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	84	2201	42	Independent claims in excess of 3
1203	280	2203	140	Multiple dependent claim, if not paid
1204	84	2204	42	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

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1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	410	2252	205	Extension for reply within second month	
1253	930	2253	465	Extension for reply within third month	
1254	1,450	2254	725	Extension for reply within fourth month	
1255	1,970	2255	985	Extension for reply within fifth month	
1401	320	2401	160	Notice of Appeal	
1402	320	2402	160	Filing brief in support of an appeal	
1403	280	2403	140	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive - unavoidable	
1453	1,300	2453	650	Petition to revive - unintentional	
1501	1,300	2501	650	Utility issue fee (or reissue)	
1502	470	2502	235	Design issue fee	
1503	630	2503	315	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	750	2809	375	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	750	2810	375	For each additional invention to be examined (37 CFR 1.129(b))	
1801	750	2801	375	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify) _____

*Reduced by Basic Filing Fee Paid

SUBTOTAL (3) ($)

SUBMITTED BY

(Complete *(if applicable)*)

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PROVISIONAL APPLICATION COVER SHEET
Additional Page

Docket Number 100997-1/US

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Number 1 of 2

ISOTHERMAL TITRATION CALORIMETRY ASSAYS

FIELD OF THE INVENTION

The present invention relates to methods of identifying and characterizing modulators of bioprocesses and biomolecules.

BACKGROUND

A major process in drug discovery involves the study of biochemical systems and the specific nature of the interaction of biomolecules with various ligands. Microcalorimetric techniques such as isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC) are widely used in understanding these interactions (Jelesarov & Bosshard, 1999, J. Mol. Recognit., 12:3-18; Ward & Holdgate, 2001, Prog. Med. Chem., 38:309-76; Sturtevant, 1987, Ann. Rev. Phys. Chem., 38:463-88).

ITC is a technique used primarily in measuring the equilibrium heat of binding of a ligand to a macromolecule (Leavitt & Freire, 2001, Curr. Op. Struct. Biol., 11:560-6; Ladbury & Chowdhry, 1996, Chem. Biol., 3:791-801; Doyle, 1997, Biotechnology, 8:31-35; Fisher & Singh, 1995, Methods Enzymol., 259:194-221). In recent years ITC has also been used in the determination of enzymatic activity and enzyme kinetic parameters such as the Michaelis-Menten kinetic parameters K_M and k_{cat} (Todd & Gormez, 2001, Anal. Biochem., 296:179-187; Cai *et al.*, 2001, Anal. Biochem., 299:19-23).

Several studies of enzymatic activities have been reported using the detection of reaction heat for measuring kinetic parameters (Lonhienne *et al.*, 2001, Biochim. Biophys. Acta, 1545:349-356; Quemard *et al.*, 1995, Biochemistry, 34:8235-8241; Silberg & Vickery 2000, J. Biol. Chem., 275:7779-7786; Prodromou *et al.*, 1999, EMBO J., 18:754-762).

The determination of the inhibition profiles of different compounds towards a target (*e.g.*, IC_{50} value, the concentration of compound at which 50% of the enzymatic activity is inhibited) is of particular interest in drug discovery. Determination of such quantities as IC_{50} and Ki (kinetically determined inhibition constant representing the potency of an enzyme inhibitor) are imperative in designing novel potent inhibitors that will lead to developing

effective drugs. Recent experiments describe the use of DSC in determining K_d (equilibrium dissociation constant) values for binding of inhibitors to MurB (Sarver *et al.*, 2002, J. Biomol. Screening, 7:21-28). ITC is also used traditionally in the determination of K_d values but not IC_{50} or Ki values (kinetically determined inhibition parameters).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a schematic representation of a calorimetric chamber.

Figure 2A shows inhibition of *E. coli* GyrAB ATPase activity by novobiocin as measured by ITC. Figure 2B shows the data fitted to an IC_{50} curve.

Figure 3A shows *E. coli* GyrAB ATPase reaction progressing to completion after each addition of substrate. Figure 3B shows the heat calibration curve obtained by the same experiment.

Figure 4A shows inhibition of *E. coli* MurC activity by β,γ-Methyleneadenosine 5'-triphosphate as measured by ITC. Figure 4B shows the data fitted to IC_{50} curve.

Figure 5 shows inhibition of *E. coli* MurB activity by (2R)-2-{2-[3-(4-*tert*-butylphenoxy)phenyl]-4-oxo-1,3-thiazolidin-3-yl} hexanoic acid) (data fitted to an IC_{50} curve).

Figure 6A shows the heat change $\Delta Q/\Delta T$ observed, in the presence (open circles) and absence of tetracycline (closed circles), upon titrating S30 extract (containing the ribosomes) in the reaction mixture (containing DNA, amino acids, and other necessary components for the reaction to occur). Figure 6B shows the difference in the heat change $\Delta(\Delta Q/\Delta T)$, observed in the presence and in the absence of tetracycline, upon titrating S30 extract in the reaction mixture.

DETAILED DESCRIPTION

The present invention provides methods for monitoring a bioprocess using isothermal titration calorimetry.

The present invention also provides methods for monitoring the activity of a biomolecule or a mixture of biomolecules using isothermal titration calorimetry. In some embodiments, the biomolecule is an enzyme.

The present invention also provides methods for identifying and/or detecting modulators of a bioprocess by monitoring changes or alterations in the reaction heat produced by the bioprocess in the presence and in the absence of test modulators.

The present invention also provides methods for identifying and/or detecting modulators of a biomolecule by monitoring changes or alterations in the reaction heat produced by the activity or action of the biomolecule in the presence and in the absence of test modulators.

The present invention also provides methods for determining inhibition profiles (such as IC_{50} values) for compounds that inhibit a bioprocess

The present invention also provides methods for determining inhibition profiles (such as IC_{50} values) for compounds that inhibit a biomolecule. In some embodiments the biomolecule is an enzyme and an inhibition profile is determined for the activity of the enzyme.

The methods of the present invention have general applicability to kinetic measurements because the methods are based upon the determination of the heat of reaction, which is a universal quantity.

The methods of the present invention do not require coupling enzymes, and are devoid of artifacts and interferences that are sometimes produced in spectrophotometric and fluorometric assays.

The methods of the present invention are particularly useful for reactions where there is no straightforward way of detecting product formation or substrate depletion.

The present invention provides screening methods for identifying a biomolecule modulator. The methods comprise incubating a plurality of test mixtures, each test mixture comprising at least one biomolecule ligand or substrate, and at least one biomolecule, under conditions effective to permit at least a portion of the biomolecule to react to a measurable extent; adding a test biomolecule modulator to at least one test mixture; detecting an activity signal of each biomolecule, as measured by a heat change, in the presence and in the absence of the biomolecule ligand or substrate; and comparing the activity signal of each biomolecule in the presence of the biomolecule modulator with an activity signal of the same biomolecule in the absence of the biomolecule modulator under the same conditions.

In one embodiment of the invention the biomolecule is a protein.

In a particular embodiment of the invention the protein is an enzyme.

In a further particular embodiment of the invention the enzyme is gyrase (GyrAB), MurB, or MurC.

In a most particular embodiment of the invention the enzyme is *Escherichia coli* gyrase (GyrAB), *E. coli* MurB, or *E. coli* MurC.

In some embodiments of the present invention detecting the activity signal comprises detecting the change in energy input required to maintain the system's thermal equilibrium

In some embodiments, the detecting the activity signal comprises detecting the change in energy input required to maintain thermal equilibrium between the test mixture and a control.

The present invention also provides methods for identifying a bioprocess modulator. The methods comprise incubating a plurality of test mixtures, each test mixture comprising at least one biomolecule having activity in the bioprocess, and at least one biomolecule ligand or substrate, wherein the conditions are effective to permit at least one biomolecule to react to a measurable extent; adding a test bioprocess modulator to at least one test mixture; detecting an activity signal of the bioprocess in the presence and in the absence of the biomolecule ligand or substrate; and comparing the activity signal of the bioprocess in the presence of the bioprocess modulator with a activity signal of the same biomolecule in the absence of the bioprocess modulator under the same conditions.

Other embodiments of the invention comprise a method for identifying compounds that modulate a bioprocess comprising: measuring heat change in the bioprocess, in the presence and in the absence of a test compound; and identifying a compound that modulates the bioprocess as one that yields an alteration in heat change in the presence of the compound relative to heat change in the absence of the compound.

Particular embodiments of the invention include methods for identifying compounds that modulate the activity of a biomolecule comprising: measuring heat change resulting from activity of the biomolecule, in the presence and in the absence of a test compound; and identifying a compound that modulates the biomolecule as one that yields an alteration in the heat change in the presence of the compound relative to the heat changein the absence of the compound.

In addition, the methods of the present invention allow for the continuous measurement of initial rates ($\Delta Q/\Delta t$ can be measured instantly as the reaction occurs). Advantages of this are that problems such as product inhibition can be minimized in determining kinetic parameters of the reaction and/or inhibition profiles of potential inhibitors. Consequently, the methods of the present invention are particularly applicable to the screening and identification of novel modulators of diverse biomolecular processes.

Typically in ITC experiments, a macromolecule is placed in the sample cell, water or buffer is placed in the reference cell, and the cells are equilibrated; a preset differential power (DP) or baseline signal is applied to the cells such that ΔT is zero or constant (Figure1). Upon reaching thermal equilibrium between the two cells, a ligand is injected into the sample cell through a titration syringe. Depending on the nature of the interaction between the macromolecule and the ligand, DP will decrease (exothermic event), or increase (endothermic) to compensate for the heat released or absorbed in the sample cell while keeping ΔT between the cells zero or constant.

In one aspect, the present invention provides methods for monitoring the inhibition of an enzyme, including methods of determining the inhibition profile of an inhibitor on an enzyme. In our approach, the cells are equilibrated after the enzymatic reaction is started and placed into the sample cell. The baseline signal DP is now lower than the preset value because the enzymatic reaction releases heat in the sample cell (exothermic reaction) in a steady state; consequently less power is needed to be applied to the sample cell to keep the two cells at the preset ΔT. During the experimental run, the inhibitor is injected into the sample cell. As the enzyme is inhibited, less heat is produced in the sample cell. The decrease in the heat produced by the enzymatic reaction per unit time results in an increase in the DP signal since more power must now be provided from the feedback heater to keep the two cells at the preset ΔT. The direction of the heat change would be opposite for an endothermic reaction.

The methods of the present invention are particularly useful for high throughput screening of enzyme inhibitors since the methods allow for the determination of the IC_{50} value in a single titration. There is no need to initiate the reaction after the system is equilibrated (the reaction is started before equilibration), and therefore there is no need for co-injection of a reaction initiator with the reaction inhibitor.

The methods of the present invention can be applied to the identification and characterization of enzyme modulators (inhibitors or activators) by a heat exchange assay. We have determined the kinetic and inhibition parameters for three different enzymatic systems. In general, monitoring of the change in heat with respect to time ($\Delta Q/\Delta t$) is used in measuring the initial rates of reactions under various conditions, including altered concentrations of enzyme, substrate, and modulator (inhibitor or activator). More specifically, we have characterized the enzymatic activity and studied modulation by known inhibitors of that enzymatic activity, for the following: the ATPase activity of *Escherichia*

coli gyrase (GyrAB), the redox activity of *E. coli* MurB (converts uridine diphosphate-*N*-acetyl-glucosamine enoylpyruvate (EP-UDP-GlcNAc) to uridine diphosphate-*N*-acetylmuramic acid (UDP-MurNAc) with the simultaneous oxidation of NADPH to $NADP^+$), and the ligase activity of *E. coli* MurC (converts UDP-MurNAc to uridine diphosphate-*N*-acetylmuramyl-L-alanine (UDP-MurNAc-Ala) with the simultaneous conversion of ATP to ADP and P_i).

As used herein, a "bioprocess" comprises one or more biomolecules, capable of catalyzing and/or supporting at least one reaction, in combination with, at a minimum, all of the necessary set of chemical reactants required for the reaction to occur. A bioprocess may comprise multiple reactions running simultaneously. Assay methods of the present invention can be used to monitor any or all of these reactions. Assay methods of the present invention can be used to screen for and identify compounds that modulate any or all of these reactions.

Assays of the present invention can be used to monitor any bioprocess that releases of absorbs heat.Assays of the present invention can be used to screen for compounds that modulate (inhibit or augment) any bioprocess.

As used herein, the terms "biomolecule" refer to chemical entities capable of catalyzing and/or supporting a chemical reaction in a bioprocess. Examples of biomolecules include, but are not limited to proteins, enzymes, polypeptides, DNA or RNA oligonucleotides, DNA or RNA polynucleotides, lipids, cofactors, and carbohydrates.

Assays of the present invention can be used to monitor the activity or action of any biomolecule-where, as a result of the activity or action, heat is released or absorbed. Assays of the present invention can be used to screen for compounds that modulate (inhibit or augment) the activity or action of any biomolecule.

In some embodiments, the bioprocess is an enzymatic reaction.

In some embodiments, the bioprocess is DNA supercoiling, the biomolecule is selected from type II topoisomerases including but not limited to DNA gyrase (GyrAB) and topoisomerase IV, and the chemical reactants are selected from adenosine triphosphate and DNA molecules including, but not limited to, oligonucleotides, PCR products, recombinant plasmids, cosmids, and chromosomal preparations.

In some embodiments, the ATPase activity of a DNA gyrase is measured as indicative of DNA supercoiling or unwinding.

In some embodiments, the ATPase activity of GyrAB measured as indicative of DNA supercoiling or unwinding.

In some embodiments, the bioprocess is a coupled process of chemical reactions comprising a metabolic pathway including, but not limited to, bacterial cell wall biosynthesis, aromatic amino acid biosynthesis, oxidative phosphorylation, citric acid cycle, glycolysis, gluconeogenesis, photophosphorylation, lipid biosynthesis, glycosylation and cofactor biosynthesis.

In some embodiments, the bioprocess is a coupled process of chemical reactions comprising a macromolecular process including, but not limited to, DNA replication, RNA polymerization, transcription/translation, protein synthesis, protein secretion, microtubule polymerization, histone deacetylation, proteosome protein degradation, chaperone mediated protein folding, and cellular respiration.

In some embodiments, the biomolecule is an enzyme.

Enzymes from any source can be monitored or modulated using the assays of the present invention, including, but not limited to, viral, bacterial, prokaryotic, eukaryotic, and cancer or disease-associated enzymes.

In some embodiments, the biomolecule is a bacterial enzyme.

In some embodiments, the biomolecule is a bacterial enzyme from *Escherichia coli, Salmonella spp, Shigella spp, Haemophilus influenzae, Moraxella catarrhalis, Pseudomonas aeruginosa, Chlamydia spp, Legionella spp, Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus pyogenes, Enterococcus faecalis, Enterococcus faecium,* or *Mycoplasma spp.*

In some embodiments, the biomolecule is gyrase (GyrAB), MurB, or MurC.

In some embodiments, the biomolecule is *Escherichia coli* gyrase (GyrAB), *E. coli* MurB, or *E. coli* MurC.

In some embodiments of the present invention, the biomolecule is an enzyme selected from oxidases/reductases that utilize at least one of the following cofactors: NADH, flavin, cobalamin, S-adensoyl methionine, ubiquinone, heme, glutathione, photooxidation, and/or iron sulfur clusters.

In some embodiments, the biomolecule is the enzyme MurB and the chemical reactants are NADH, flavin, and UDP-GlcNAc-EP.

In some embodiments, the biomolecule is an ATP-dependent ligase, including, but not limited to, the enzymes FolC, MurC, MurD, MurE, MurF.

In some embodiments, the biomolecule is a kinase, including, but not limited to, protein serine/threonine kinases, protein tyrosine kinases, or dual specificity kinases.

In some embodiments, the biomolecule is a phosphatase, including, but not limited to, pyrophosphatase, protein phosphatases, and phosphodiesterases.

In some embodiments, the biomolecule is a transmembrane signaling protein, including, but not limited to, bacterial and eukaryotic two component regulatory systems, G-protein coupled receptor-G protein complexes, and JAK-STAT signaling complexes.

In some embodiments, the biomolecule is a protein chaperone, including, but not limited to, the heat shock proteins GroEL-GroES, DnaK.

The methods of the present invention utilize the measurement of heat change as aneffect of a bioprocess. Heat change can be measured by a variety of techniques known to those of skill in the art.

In some embodiments of the present invention, heat change is measured using instruments designed for calorimetry. Such calorimeters, are known to the art and include commercially available instruments such as Microcal's isothermal titration calorimeter (Microcal VP ITC), and the differential scanning calorimeter (Microcal High-throughput DSC). In general, the activity of a bioprocess, in the presence and absence of test compounds and/or chemical reactants, is measured by monitoring the change in heat relative to a reference cell containing a control sample. Test compounds and/or chemical reactants can be introduced into sample and/or reference cells by various methods known to those skilled in the art, including, but not limited to, injection using one or more syringes.

In some embodiments, the sample and/or reference cell comprises a set of solution reservoirs on a thermal sensitive matrix, and the test compounds and/or chemical reactants are introduced using electrosmotic or vacuum-driven flow.

The sample cell can be in a variety of formats, including but not limited to, a section of capillary tubing, a set of solution reservoirs on a thermal sensitive matrix, a chamber enclosed in an adiabatic environment, etc.

The reference cell can be in a variety of formats, including but not limited to, a section of capillary tubing, a set of solution reservoirs on a thermal sensitive matrix, a chamber enclosed in an adiabatic environment, etc.

In some embodiments, heat change is measured using instruments designed for differential scanning calorimetry, and the heat change from chemical reactions resulting from a mixture of a test compound and a solution containing a bioprocess is measured in a sample cell over a temperature range relative to a control sample in a reference cell measured under the same conditions.

In some embodiments, the test compounds and/or chemical reactants are mixed and introduced into the sample cell using robotic liquid handling equipment.

In some embodiments, heat change is measured using a calorimeter selected from a Microcal ITC, a Microcal VP DSC, and a Microcal High throughput DSC.

The invention is further illustrated by way of the following examples, which are intended to elaborate several embodiments of the invention. These examples are not intended to, nor are they to be construed to, limit the scope of the invention. It will be clear that the invention may be practiced otherwise than as particularly described herein. Numerous modifications and variations of the present invention are possible in view of the teachings herein and, therefore, are within the scope of the invention.

EXAMPLES

Example 1. Inhibition of gyrase by novobiocin as measured by ITC.

The enzyme comprising *E. coli* GyrA and GyrB subunits, at a concentration of 1 μM each, were incubated in 50 mM Tris (pH 7.5), 75 mM ammonium acetate, and 200 nM of 250 base pairs (bp) DNA at room temperature for 30 minutes. The enzyme stock was diluted 20-fold to a final concentration of 50nM in assay buffer (50 mM Tris pH 7.5, 75 mM ammonium acetate, 5% glycerol, 0.5 mM EDTA, 5.5 mM magnesium chloride) that contained ATP at a final concentration of 500 μM. The diluted enzyme solution was placed in the ITC sample cell and the calorimetric cells were equilibrated. Novobiocin was diluted in assay buffer at a final concentration of 2.8 μM and it was loaded into the titration syringe. The titration was started manually after the DP signal was stabilized. A total of 15 injections of 5 μl each were performed. Time spacing in between injections was set at 120 sec. Control experiments were performed under identical conditions with experiment assay buffer in the syringe. In these experiments, no decrease in DP baseline was observed after the completion of each injection peak. In separate control experiments the linearity of the reaction was checked under the same assay conditions. The reaction progress was found to be linear with time for at least 30 minutes.The inhibition curve of the ATPase activity of *E. coli* GyrAB by novobiocin, a potent natural-product gyrase inhibitor, was generated by the ITC technique (see Figures 2A and 2B). The reaction was started by adding ATP at a final concentration of 250 μM to a mixture of *E. coli* GyrAB and a 250 bp DNA. The final concentration of *E. coli* GyrAB in the reaction was 50 nM of each subunit and the DNA concentration was 10nM. All measurements contained 50 mM Tris, pH 7.5, 75 mM ammonium acetate, 5% glycerol, 0.5

mM EDTA, and 5.5 mM magnesium chloride. Novobiocin was dissolved in the same buffer at a final concentration of 2.8 μM. The reaction was placed in the sample cell and the cells were equilibrated. Novobiocin was added in the titration syringe. Fifteen 5 μl injections were performed.

Upon novobiocin addition, the power baseline decreased after each injection peak (solid black line in Figure 2A), an observation consistent with inhibition of enzyme activity. After a certain injection, the power baseline plateaus, an indication that all enzyme activity has been inhibited. The data were analyzed as follows: the power value (μcal/sec) after each injection is subtracted from the initial power value (before any injection occurs). Each point is then calculated as % inhibition assuming that 100% inhibition occurs where the power baseline plateaus after the n^{th} injection of inhibitor. After data analysis, an IC_{50} value of 26 nM was obtained for novobiocin inhibition. This value is in close agreement with the value obtained by using an independent assay of enzyme activity using phosphate detection. (IC_{50} = 28 nM, see Table 1).

It is also possible to convert the enzyme activity expressed as power units to activity expressed as turnover number units by using a calibration curve that converts $\Delta Q/\Delta t$ to $\Delta P/\Delta t$ and calculate the % inhibition as described above. An example of such conversion is shown in Figures 3A and 3B.

The experiment shown in Figure 3 was run as follows: reconstituted *E. coli* GyrAB (a mixture of 100 nM of each GyrA, and GyrB subunits in the presence of 20 nM of a 250bp DNA) was added into the sample cell. ATP was dissolved in the same buffer at a final concentration of 145 μM and added to the titration syringe. Four injections (1, 3, 5, and 8 μl) were completed. The injection spacings were adjusted so the power baseline returned to the initial position between injections, an indication that the reaction was completed for each injection of substrate (see Figure 3A). The area under each peak represents the total heat released for the conversion of the added ATP to ADP and Pi. By plotting the concentration of ATP added in each injection vs. area of the injection peak it is possible to obtain a calibration curve the slope of which is the conversion factor $\Delta Q/\Delta P$ (heat released in μcal)/nmole of product formed). The slope of the curve shown in Figure 3B was calculated to be 11kcal/mole. This number is consistent with the number of 13 kcal/mole reported for the energy released in ATP hydrolysis. Measuring kinetic parameters such as $K_{M,\ ATP}$, k_{cat} and $K_{i,\ ADP}$ by ITC contributed to further characterization of *E. coli* GyrAB. These values are

compared with those obtained by an independent assay of enzyme activity using phosphate detection (Table 1).

Table 1 shows kinetic parameters of the *E. coli* GyrAB ATPase reaction obtained by ITC. For comparison, values obtained by an independent method of phosphate detection are included.

Table 1

Method	$K_{M, ATP}$ (μM)	$K_{i, ADP}$ (μM)	Novobiocin IC_{50} (μM)
ITC	166	18	0.026
Phosphate detection	145	18	0.028

Example 2. MurC.

E. coli MurC was diluted in assay buffer (50 mM Tris pH 8.0, 1 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP), 0.01 % (w/v) Triton X-100, 20 mM ammonium formate) to a final concentration of 10 nM. The buffer also contained the MurC substrates at the following concentrations in the ITC sample cell: 100 μM UDP-MurNAc, 275 μM L-alanine, and 300 μM ATP. β,γ-Methyleneadenosine 5'-triphosphate (AMP-PCP) was dissolved in assay buffer at a final concentration of 2.8 mM and it was loaded into the titration syringe. The titration was started manually after the DP signal was stabilized. A total of 16 injections (2, 2, 4, 4, 4, 4, 8, 8, 16, 16, 32, 32, 32, 32, 32, and 32 μl) were performed. Time spacing in between injections was set at 120 sec for all injections.

The inhibition of MurC by AMP-PCP is shown in Figure 4. All the reactions were carried in 50 mM Tris, pH 8.0, 1 mM TCEP, 0.01 % (w/v) Triton, and 20 mM ammonium formate. The final concentration of *E. coli* MurC was 10 nM. The final concentrations of the MurC substrates were as follows: 100 μM uridine diphosphate-*N*-acetylmuramic acid (UDP-MurNAc), 275 μM L-alanine, and 300 μM ATP. The reaction was initiated with the addition of MurC and placed into the sample cell. AMP-PCP was dissolved in the buffer described above at a final concentration of 2.8 mM and placed into the titration syringe.

The raw ITC data are shown in Figure 4A. An IC_{50} value of 27 μM was obtained for MurC inhibition by AMP-PCP (see Figure 4B). This value compared well with the value of 40 μM obtained using an independent assay of enzyme activity by detecting inorganic phosphate formation.

Other kinetic measurements were performed to further characterize *E. coli* MurC by ITC. Table 2 shows kinetic parameters of the *E. coli* MurC reaction obtained by ITC. For comparison, values obtained by an independent method of phosphate detection are included.

Table 2

Method	$K_{M, ATP}$ (μM)	$K_{M, UNAM}$ (μM)	$K_{M, ALA}$ (μM)	k_{cat} (min^{-1})	AMP-PCP IC_{50} (μM)
ITC	61	21	44	50	27
Phosphate detection	101	19	56	71	35

Example 3. MurB.

E. coli MurB was diluted in assay buffer (50 mM Tris pH 8.0, 1 mM TCEP, 20 mM KCl, 0.005 % Triton X-100) to a final concentration of 1.3 nM. The buffer contained the MurB substrates at the following concentrations in the ITC sample cell: 100 μM NADPH and 60 μM EP. DMSO was also added at a final concentration of 2.7 % (v/v). The compound ((2R)-2-{2-[3-(4-*tert*-butylphenoxy)phenyl]-4-oxo-1,3-thiazolidin-3-yl} hexanoicacid) stock was prepared in DMSO and diluted in assay buffer at a final concentration of 2.8 mM. The titration was started manually after the DP signal stabilized and a total of 10 injections (8, 8, 17, 17, 24, 24, 32, 32, and 50 μl) were performed. Time spacing in between injections was set at 100, 100, 120, 120, 120, 150, 150, 150, and 180 sec respectively.

The inhibition of *E. coli* MurB by (2R)-2-{2-[3-(4-*tert*-butylphenoxy)phenyl]-4-oxo-1,3-thiazolidin-3-yl} hexanoic acid) (a 4-thiazolidinone compound)was studied by the same method. Reactions were carried in 50 mM Tris, pH 8.0, 1 mM TCEP, 20 mM KCl, 0.005 % Triton, in the presence of 100 μM NADPH and 60 μM EP. The concentration of MurB was 1.3 nM. An IC_{50} value of 154 μM was obtained by the method described above (see Figure 5). An IC_{50} value of 101 μM was obtained by an absorbance method of measuring MurB activity by measuring NADPH depletion at 340 nm.

Other kinetic measurements were performed to further characterize *E. coli* MurB by ITC. Table 3 shows kinetic parameters of the *E. coli* MurB reaction obtained by ITC. For comparison, values obtained by an independent method of NADPH depletion detection are included.

Table 3

Method	$K_{M, NADPH}$ (μM)	$K_{M, EP}$ (μM)	4-thiazolidinone analogue IC_{50} (μM)

ITC	9	8	154
Absorbance	9	6	101

Example 4. Inhibition of the transcription/translation process by tetracycline as measured by ITC.

The inhibition of the transcription/translation process by tetracycline is shown in figure 6.

A reaction mix was prepared, containing 17.5 mM Tris acetate, 95.2 mM potassium acetate, 15 mM ammonium acetate, 5mM TCEP, 5 mM ATP, 1.25 mM each of four nucleotide triphospates, adenosine triphosphate (ATP), cytidine triphosphate (CTP), guanidine triphosphate (GTP), and uridine triphosphate (UTP), 87 mM phospho(enol)pyruvate (PEP), 0.25 mg/ml transfer ribonucleic acid (tRNA), 350 mg polyethyleneglycol (PEG), 0.05 mg/ml folinic acid, 2.5 mM cyclic adenosine monophosphate (cAMP), 2mM isopropyl b-D-1-thiogalactopyranoside (IPTG), and 18.9 mM each of the 20 amino acids.

A 2 ml substrate mixture was prepared, containing 800 µl of the reaction mixture, 15 µl of 1 mg/ml pBEST *luc*™ vector DNA (Promega) and 1185 µl H_2O.

The same substrate mixture was prepared, containing containing 800 µl of the reaction mixture, 15 µl of 1 mg/ml pBEST *luc*™ vector DNA (Promega), 20 µl of 20 mM tetracycline in dimethyl sulfoxide (DMSO), and 1165 µl H_2O.

S30 extract (containing ribosomes and a wide variety of enzymes and cofactors needed for the transcription/translation process) from bacterial lysate was prepared and added in the titration syringe. S30 extract was titrated in the sample cell containing the substrate mixture. 10 injections, 10 µl each were performed Initially upon S30 extract addition the power baseline increased after each injection (indicative of an endothermic event). At the fifth injection of S30 extract the power baseline decreased and continued decreasing after subsequent injections (indicative of exothermic event) (see Figure 6A, closed circles).

S30 extract was titrated in the sample cell containing the substrate mixture and tetracycline (see Figure 6A, open circles). Initially the same heat changes were observed, but at the fifth injection of S30 extract (exothermic event starts to take place), differences in the magnitude of the heat change were evident. These differences were calculated and plotted as differences in the heat change observed $\Delta(\Delta Q/\Delta T)$ upon titrating S30 extract in the substrate mixture in the presence or absence of tetracycline (see Figure 6B). $\Delta(\Delta Q/\Delta T)$ is defined as the heat change $\Delta Q/\Delta T$ observed in the presence of tetracycline minus the heat change

$\Delta Q/\Delta T$ observed in the absence of tetracycline. It is evident from figure 6B that a portion of the transcription/translation process is inhibited by tetracycline since at the fifth injection of S30 extract the heat change is smaller in the presence of tetracycline (negative $\Delta(\Delta Q/\Delta T)$) than that in the absence of tetracycline. The effect of tetracycline in the heat change of the process becomes less evident upon continuing S30 injections (the differences in the heat change observed in the presence and absence of tetracycline become smaller, or $\Delta(\Delta Q/\Delta T)$ returns to 0).

The foregoing examples are meant to illustrate the invention and are not to be construed to limit the invention in any way. Those skilled in the art will recognize modifications that are within the spirit and scope of the invention.

We claim:

1. A screening method for identifying a biomolecule modulator, the method comprising:

a) incubating a plurality of test mixtures, each test mixture comprising at least one biomolecule ligand or substrate, and at least one biomolecule, under conditions effective to permit at least a portion of the biomolecule to react to a measurable extent;

b) adding a test biomolecule modulator to at least one test mixture;

c) detecting an activity signal of each biomolecule as measured by a heat change in the presence and in the absence of the biomolecule ligand or substrate; and

d) comparing the activity signal of each biomolecule in the presence of the biomolecule modulator with a activity signal of the same biomolecule in the absence of the biomolecule modulator under the same conditions.

2. The method of claim 1 wherein the biomolecule is a protein.

3. The method of claim 1 wherein the biomolecule is an enzyme.

4. The method of claim 3 wherein the enzyme is gyrase (GyrAB), MurB, or MurC.

5. The method of claim 3 wherein the enzyme is *Escherichia coli* gyrase (GyrAB), *E. coli* MurB, or *E. coli* MurC.

6. The method of claim 4 wherein the enzyme is GyrAB.

7. The method of claim 6 further wherein ATPase activity is measured.

8. The method of claim 1 wherein detecting the activity signal comprises detecting the change in energy input required to maintain thermal equilibrium between the test mixture and a control.

9. The method of claim 1, adapted for high throughput screening.

10. The method of claim 1, wherein the method is carried out in a calorimeter.

11. A screening method for identifying a bioprocess modulator, the method comprising:

a) incubating a plurality of test mixtures, each test mixture comprising at least one biomolecule having activity in the bioprocess, and at least one biomolecule ligand or substrate, wherein the conditions are effective to permit at least one biomolecule to react to a measurable extent;

b) adding a test bioprocess modulator to at least one test mixture;

c) detecting an activity signal of the bioprocess in the presence and in the absence of the biomolecule ligand or substrate; and

d) comparing the activity signal of the bioprocess in the presence of the bioprocess modulator with a activity signal of the same biomolecule in the absence of the bioprocess modulator under the same conditions.

12. The method of claim 11 wherein the bioprocess is transcription/translation.

13. A method for identifying compounds that modulate a bioprocess comprising:

a) measuring heat change in the bioprocess, in the presence and in the absence of a test compound; and

b) identifying a compound that modulates the bioprocess as one that yields an alteration in heat change in the presence of the compound relative to heat change in the absence of the compound.

14. A method for identifying compounds that modulate the activity of a biomolecule comprising:

a) measuring heat change resulting from activity of the biomolecule, in the presence and in the absence of a test compound; and

b) identifying a compound that modulates the biomolecule as one that yields an alteration in heat change in the presence of the compound relative to heat change in the absence of the compound.

ABSTRACT

An isothermal analysis method for measuring the activity of modulators of biomolecules.

FIGURE 1

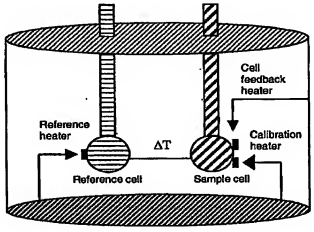

FIGURE 2A

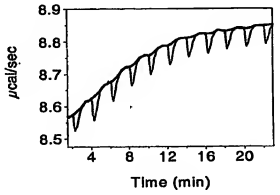

FIGURE 2B

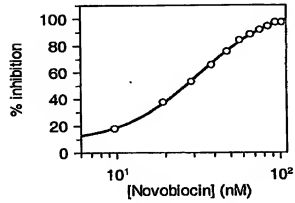

FIGURE 3A

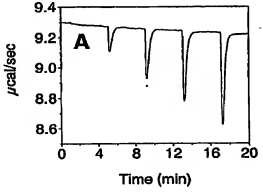

FIGURE 3B

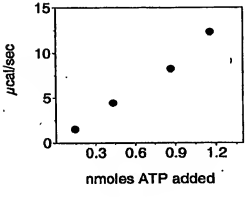

FIGURE 4A

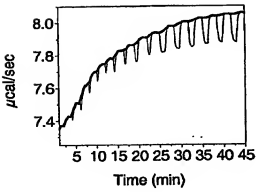

FIGURE 4B

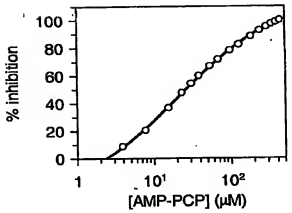

FIGURE 5

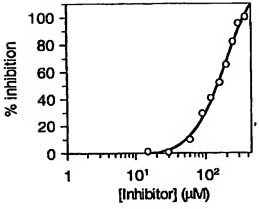

FIGURE 6A

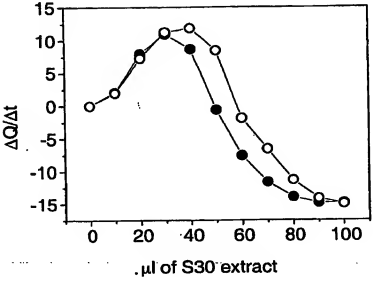

FIGURE 6B